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Amphotericin B–sterol complex formation and competition with egg phosphatidylcholine: a monolayer study

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The conditions of formation of amphotericin B–cholesterol or –ergosterol complexes in monolayers are investigated by the penetration into a monolayer of egg phosphatidylcholine/sterol of ^{14}C-labelled *N*-fructosyl-amphotericin B dissolved in the aqueous subphase. An increase of both surface pressure and radioactivity as a function of concentration are observed simultaneously while a 'saturation' effect occurs only for the surface pressure. The experiments are not accurate enough to make conclusions about the number of actually penetrated amphotericin B molecules. Therefore, the existence of an amphotericin B–sterol complex was evidenced from a study of surface pressure area per molecule isotherm. The results indicate that a complex with a 2:1 stoichiometry is formed and that the amphotericin B–ergosterol interaction is larger than the amphotericin B–cholesterol interaction. The complex is dissociated by addition of egg phosphatidylcholine due to a competition between egg phosphatidylcholine and amphotericin B for sterol.

Introduction

Amphotericin B, a polyene macrolide, is an antibiotic ionophore known to interact with cell plasma membranes, inducing a large permeability to ions and small non-electrolytes, eventually leading to cell death. The action of amphotericin B depends on the presence of sterol. It has been shown, with both biological membranes and their lipidic models, that ergosterol-containing membranes are more sensitive than cholesterol-containing ones, which is the basis for its use in antifungal chemotherapy [1]. This selective toxicity for ergosterol-containing membranes is rather poor for amphotericin B itself but has been found to be greater for the amphotericin B derivatives whose carboxyl group in the C-18 position on the macrolide ring is blocked by substitution, such as amphotericin B methyl ester [2,3].

The mechanism of action of polyene at the membrane level is poorly understood. Two mechanisms have been proposed accounting for the necessity of sterol. In the first one, the action of sterol is indirect. Indeed the interaction between polyene and membranes would require a particular ordered state in the monolayer and the penetration of polyene molecules would be facilitated by phase boundary defects, both phenomena induced by sterol [4–6]. The second mechanism, most generally accepted, is the formation of

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amphotericin B–sterol complexes which would be organized in channels [7,8]. Polyene–sterol complexes have been demonstrated and their stoichiometry defined in the case of filipin, a small ring polyene [9]. However, in the case of amphotericin B or nystatin evidence of a complex is not clear [10]. The stoichiometry experimentally found in the literature varies from 1 : 4 to 1 : 0.7 because the various methods used do not allow the distinction between amphotericin B molecules that have actually penetrated into the phospholipid bilayer and molecules only adsorbed on the polar surface of the layer. Monolayer studies appear to be a useful approach to this question. Demel and coworkers [11,12] first measured the effects of various polyenes on the surface pressure of lipidic monolayers. Ockman [13], studying polarized absorption spectra, was able to relate the surface pressure effect of amphotericin B on monolayers to its orientation and extent of penetration.

In this investigation, the actual penetration of polyene in monolayers was directly measured using two ^{14}C-labelled derivatives of amphotericin B, fructosyl-amphotericin B whose physicochemical and biological properties are very similar to those of amphotericin B itself [3] and fructosyl-amphotericin B methyl ester, a very close analog of amphotericin B methyl ester. In order to ascertain the existence of amphotericin B–sterol complexes as well as to estimate their stoichiometry inside the membrane, a systematic study of surface pressure versus area per molecule on monolayers were made from various binary mixtures (amphotericin B and sterol), as well as ternary mixtures (amphotericin B, sterol, egg phosphatidylcholine) and interpreted in terms of classical thermodynamics of monolayers of defined composition. The results of this study define more precisely the mechanism of polyene interaction with membrane and the conditions in which polyene–sterol complexes form.

Material and Methods

Antibiotics and derivatives

Amphotericin B was a Squibb & Son (Princeton, NY) product and its derivative amphotericin B methyl ester was prepared by methods described previously [14]. Radiolabelled (^{14}C) amphotericin B derivatives *N*-fructosyl-amphotericin B and *N*-fructosyl-amphotericin B methyl ester were prepared according to a previously described method [15] using [^{14}C]glucose (uniformly labelled) and checked for purity by chromatography. The specific activity was 14 mCi/mmol.

Lipids

L-α-Phosphatidylcholine was prepared from egg yolk according to the method of Patel and Sparrow [16].

Cholesterol and ergosterol were supplied by Fluka. Both sterols were recrystallized twice in ethanol before use.

Methods

The radioactivity emitted by the ^{14}C-labelled amphotericin B derivative was monitored using a gas flow (90% argon, 10% methane) counter. The β emission of ^{14}C (0.56 MeV) has a mean free path of 300 μm. The measurements were carried out as follows: [^{14}C]glucose of the same specific activity and concentration was introduced in the subphase together with radioactive polyene derivatives. The number, n, of counts attributable to polyene was taken as $n = C_T - C_G$ where C_T and C_G are respectively the total number of counts and the number of counts for glucose alone. n was measured within 95% confidence limits ($n > 2\sqrt{C_T} + 2\sqrt{C_G}$). Calibration was obtained using a known amount of radioactive material spread dry on metallic surfaces of known area. From these values, the number, δ, of polyene molecules per cm^2 was calculated. A known amount of sterol/egg phosphatidylcholine was spread from a solution in chloroform/ethanol, on 100 ml of an aqueous subphase of 35 cm^2 area, up to a given pressure (P_0 = initial pressure). The polyene solution (in dimethylformamide) was introduced in microliter quantities through a side arm in the subphase which is continuously stirred with a teflon-coated magnetic stirrer. Then, the variation of the surface pressure due to penetration of amphotericin B in the monolayer was determined from surface tension measurements (Wilhelmy wettable plate method) using a platinum blade connected to a force transducer (Hewlett Packard). P and δ were measured simultaneously on the same film.

For isotherms of mixtures the surface pressure, P, was directly measured by means of a displacement transducer (Kaman) using a Langmuir through with an area of about 300 cm^2 designed in the laboratory. The spreading was made from the same organic solution as above.

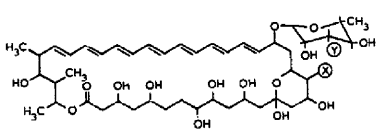
Experimental results

Amphotericin B shown in Fig. 1 is an amphiphilic and zwitterionic molecule. When the carboxyl group is esterified by a methyl group, the molecule is positively charged and is called amphotericin B methyl ester. The radiolabelling by a fructosyl group increases slightly the solubility in water of fructosyl-amphotericin B with respect to amphotericin B, but the ionophoric biological properties of the two molecules are equivalent [3].

Penetration experiments

The surface pressure, P, and the number, δ, of polyene molecules per cm^2 were measured as a function of polyene concentration in the subphase, which was increased by successive additions from $8 \cdot 10^{-7}$ to 10^{-5} mol $\cdot l^{-1}$. Preliminary experiments showed that δ values become stable after 30 min following polyene addition in the subphase and did not vary significantly during 15 h, indicating the absence of long-term reorganization.

In Fig. 2 the results obtained with fructosyl-amphotericin B in cholesterol/ and ergosterol/egg-phosphatidylcholine mixed monolayers (20 : 80 mol/mol) are plotted. The initial pressure, P_0, before polyene addition was 12 ± 0.1 mN $\cdot m^{-1}$.



Compound	X	Y	Net electric charge (pH 7)
Amphotericin B	COO^-	NH_3^+	0 (±)
Amphotericin B methyl ester	$COOCH_3$	NH_3^+	+
Fructosyl-amphotericin B	COO^-	$NH_2{-}C_6O_5H_{11}$	0 (±)
Fructosyl-amphotericin B methyl ester	$COOCH_3$	$NH_2{-}C_6O_5H_{11}$	+

Fig. 1. Structure of amphotericin B (AMB) and its derivatives.

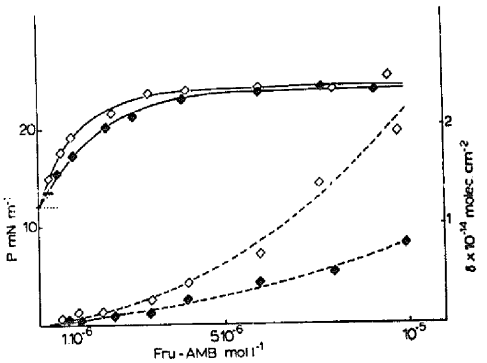


Fig. 2. Evolution of the surface pressure, P, and of the number, δ, of polyene molecules adsorbed per cm^2 with increasing polyene concentration in the subphase in mixed monolayers. Open symbols, cholesterol/egg phosphatidylcholine 20 : 80; closed symbols, ergosterol/egg phosphatidylcholine 20 : 80; P, solid lines; δ, dotted lines. Fru AMP, fructosyl-amphotericin B.

In this figure, P (left-hand-side ordinate) and δ (right-hand-side ordinate) are plotted versus fructosyl-amphotericin B concentration in the subphase. In both cholesterol- and ergosterol-containing monolayers, the surface pressure P increases rapidly with fructosyl-amphotericin B concentration and reaches a maximum value of 22 ± 1 mN $\cdot m^{-1}$ for a concentration of about $2 \cdot 3 \cdot 10^{-6}$ mol $\cdot l^{-1}$. On the other hand, the number of polyene molecules in the interfacial layer increases continuously, well beyond the concentration at which P itself becomes constant, independent of concentration.

Similar behaviour was observed both qualitatively and quantitatively for fructosyl-amphotericin B methyl ester, which is positively charged. The maximum P (22 mN $\cdot m^{-1}$) value reached was approximately the same as with fructosyl-amphotericin B at the same concentration in the subphase. However, δ values were found to be different for the two polyenes. At the same $4 \cdot 10^{-6}$ mol $\cdot l^{-1}$ concentration in the subphase (at which in both cases P was already maximum), δ were equal to $(0.38 \pm 0.01) \cdot 10^{14}$ and $(0.22 \pm 0.01) \cdot 10^{14}$ molecules $\cdot cm^{-2}$ (Fig. 2) for fructosyl-amphotericin B in cholesterol- and ergosterol-containing monolayers respectively, whereas the corresponding values for fructosyl-amphotericin B methyl

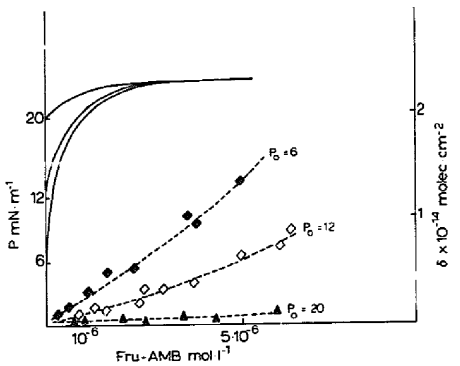


Fig. 3. Evolution of the surface pressure P and of the number δ of polyene molecules adsorbed per cm^2 with increasing polyene concentration in the subphase on a cholesterol/egg phosphatidylcholine mixed monolayer (20:80), starting from initial surface pressures of 6, 12 and 20 $mN \cdot m^{-1}$. P, solid lines; δ, dotted lines. Fru-AMB, fructosyl-amphotericin B.

ester were $(0.15 \pm 0.01) \cdot 10^{14}$ and $(0.40 \pm 0.01) \cdot 10^{14}$ (curves not represented). δ was shown to be strongly dependent on the initial surface pressure of the monolayer. In Fig. 3 are plotted the results obtained on cholesterol/egg phosphatidylcholine monolayers whose P_0 values were 6, 12 and 20 $mN \cdot m^{-1}$ upon addition of increasing concentrations of fructosyl-amphotericin B. Whatever its initial value, the evolution of P with polyene concentration was the same and reached the same maximum value at the same polyene concentration. The evolution of the curves as a function of the concentration was the same in all three cases but the δ values were quantitatively very different: the higher the P_0, the lower the δ values. At $4 \cdot 10^{-6}$ mol $\cdot l^{-1}$, δ values were $(0.02 \pm 0.02) \cdot 10^{14}$ (P_0: 20 $mN \cdot m^{-1}$), $(0.40 \pm 0.05) \cdot 10^{14}$ (P_0: 12 $mN \cdot m^{-1}$) and $(1.10 \pm 0.05) \cdot 10^{14}$ (P_0: 6 $mN \cdot m^{-1}$).

δ is not very sensitive to the sterol concentration in monolayers, as shown in Table I, in which δ values obtained for fructosyl-amphotericin B methyl ester and fructosyl-amphotericin B at the same concentration ($4 \cdot 10^{-6}$ mol $\cdot l^{-1}$) in the subphase are given for monolayers containing from 0 to 100 mol% cholesterol. δ values are not significantly different from 20 to 80 mol%. They are smaller by a factor of two in sterol-free monolayers and larger by the same factor in pure cholesterol monolayers. The penetration in mixed egg-phosphatidylcholine monolayers of the two amphotericin B derivatives, fructosyl-amphotericin B, whose physicochemical properties and biological activity are quite similar to amphotericin B, and fructosyl-amphotericin B methyl ester, appears quite comparable. In both cases, penetration increases linearly in relation to with polyene concentration in the subphase and does not depend upon sterol concentration. Moreover, the quantitative differences between the penetration of the two polyenes in ergosterol- and cholesterol-containing monolayers are not very important. Within the limit of accuracy of radioactive measurements, there is no indication of any preferential sterol–polyene interaction. The prominent features of the penetration are that it strongly depends on the initial pressure of the film and that this penetration increases with polyene concentration well beyond the point where the pressure of the monolayer has reached its maximum value.

The radioactivity measurements cannot make the distinction between molecules that have actually penetrated or molecules only adsorbed. In order to obtain more information about the behaviour of polyenes in monolayers, systematic and more precise determinations of pressure versus area per molecule isotherms (P-A) were carried out.

Isotherms of mixed monolayers

From surface pressure measurements we studied mixed monolayers of amphotericin B with mem-

TABLE I

BINDING TO MIXED MONOLAYERS AS A FUNCTION OF CHOLESTEROL CONTENT

X = cholesterol/cholesterol + egg phosphatidylcholine; EPC + chol = egg phosphatidylcholine + cholesterol (molec $\cdot cm^{-2} \times 10^{14}$); Fru-AMB = fructosyl-amphotericin B (molec $\cdot cm^{-2} \times 10^{14}$); Fru-AME = fructosyl-amphotericin B methyl ester (molec $\cdot cm^{-2} \times 10^{14}$).

X	EPC + chol	P ($mN \cdot m^{-1}$)	Fru-AMB	Fru-AME
0	1.16	21 ±0.5	0.16 ± 0.01	0.12 ± 0.01
20	1.46	21.5 ± 0.5	0.38 ± 0.01	0.15 ± 0.01
50	1.98	21.5 ± 0.5	0.32 ± 0.01	0.14 ± 0.01
72	2.28	21 ±0.5	0.38 ± 0.01	0.18 ± 0.01
83	2.4	22 ±0.5	0.31 ± 0.01	0.24 ± 0.01
100	2.6	30 ±0.5	0.70 ± 0.01	0.49 ± 0.01

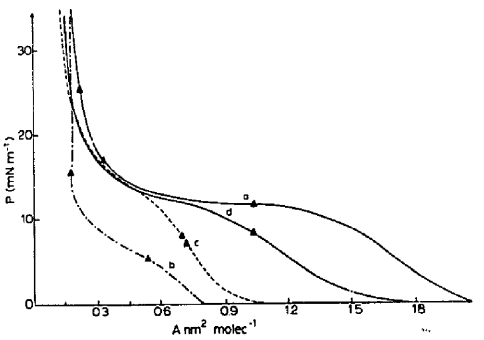


Fig. 4. Surface pressure versus area per molecule isotherms of amphotericin B (AMB), spread on 10^{-1} mol·l^{-1} NaCl at pH 5.6 and $t = 22°C$. (a) First rapid compression; (b) decompression; (c) second rapid compression; (d) first slow compression.

brane components. The existence of an eventual amphotericin B–sterol complex and its stoichiometry were deduced from the study of amphotericin B/sterol mixtures. The stability of the amphotericin B–sterol complex in the membrane and eventual competition with other membrane components were investigated using egg phosphatidylcholine/amphotericin B/sterol, egg phosphatidylcholine/amphotericin B and egg phosphatidylcholine/sterol mixtures.

Amphotericin B/sterol mixtures. The behaviour of the monolayer is rather typical of a rigid molecule for sterol but not for amphotericin B. Therefore the properties of pure amphotericin B were investigated. Fig. 4 shows the surface-pressure area per molecule isotherms (*P-A*) of amphotericin B spread on an aqueous solution of 10^{-1} mol·l^{-1} NaCl at pH 5.6 and $t = 22°C$. During a rapid compression (Fig. 4, curve a) at 0.1 nm^2/molecule per min, the lift-off occurs at approx. 2 nm^2/molecule. Then, a plateau region occurs from approximately 0.5 to 1.0 nm^2 depending upon the rate of compression. Above the plateau region the slope increases precipitously and the limiting area per molecule was calculated to be approximately 0.25 nm^2. During decompression (curve b), the pressure decreases rapidly and reaches zero at an area below 0.8 nm^2. If the same monolayer is compressed again (curve c) the lift-off is at about 1.1 nm^2 while at very high pressure the limit area per molecule is smaller than that in the first compression and is approx. 0.15 nm^2. When the isotherm is recorded with a slow compression rate (0.05 nm^2/molecule per min, curve d), the plateau length is reduced and the area per molecule reached at very high pressure is smaller than in the case of a rapid compression.

An evaluation of the cross-section of a molecule estimated from molecular models gives a value of about 1.8 nm^2, when the polar moiety of the macrolide ring of amphotericin B is in contact with water and the hydrophobic chain outside (lying form), and 0.55 nm^2 when both of them are perpendicular to the air/water interface (erect form). Whatever the compression rate may be, at very high pressure the limiting area per molecule is smaller than the minimum cross-section of the molecule, indicating that some desorption occurs, due to the high solubility of the numerous polar groups in water. The plateau could be due to a progressive change in the orientation of the molecule, from a lying to an erect form, and to the desorption of the molecule into the water.

The *P-A* isotherms were recorded for amphotericin B/cholesterol mixtures spread on 10^{-1} mol·l^{-1} NaCl at pH 5.6 (Fig. 5). Similar curves were obtained with amphotericin B/ergosterol mixtures (data not shown). For a given pressure the areas per molecule at the same mole fraction were larger with ergosterol than with cholesterol. At high mole

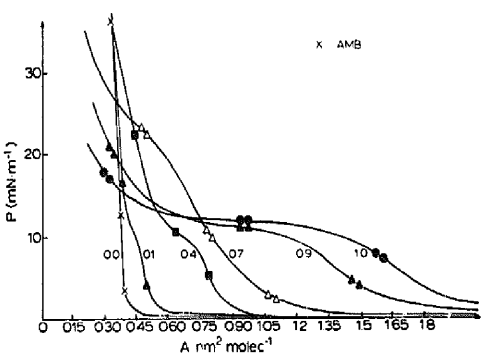


Fig. 5. Surface pressure versus area per molecule isotherms of amphotericin B (AMB)/cholesterol mixed monolayer spread on 10^{-1} mol·l^{-1} NaCl at pH 5.6 and $t = 22°C$. The curves were continuously recorded. The symbols are used only to designate the composition of the films (X = mole fraction). For the sake of clarity only selected isotherms are given.

fraction of amphotericin B the curves were similar to those obtained with pure amphotericin B, with a similar plateau region and desorption at high pressure. At low mole fraction the curves were similar to those obtained with pure cholesterol. Within the mole fraction range 0.6–0.7, isotherms were characteristic and very different from the others, without a plateau region, with only a shoulder at about 20 $mN \cdot m^{-1}$, without desorption until this pressure, which could indicate some interaction for a stoichiometry of 0.66.

In order to reveal the interaction between the two components of the mixtures, the mean area per molecule was plotted as a function of the mole fraction of amphotericin B at a given pressure. In these plots a straight line corresponds to additivity and any deviation from this line corresponds to an interaction at two or three dimensions.

Two extreme pressures were chosen (Fig. 6): 5 $mN \cdot m^{-1}$, for which amphotericin B molecules are in lying form, and 20 $mN \cdot m^{-1}$, a pressure close to the equilibrium pressure in the lamellar phases model of the biological membrane, for which the molecules are in 'erect' form. At $P = 5$ $mN \cdot m^{-1}$ the additivity rule is not observed with either cholesterol (Fig. 6a) or ergosterol (Fig. 6b). A change in the slope was observed between the mole fraction 0.6 and 0.7. The explanation could be that amphotericin B molecules are in the 'lying' form at high amphotericin B mole fractions and 'erect' form below a mole fraction equal to 0.6, because in this region the area per molecule for amphotericin B is extrapolated to be much smaller. Thus, with sterol, even at low pressure, amphotericin B is shown to be in the erect form. The deviation from the straight line is larger for ergosterol than for cholesterol. At $P = 20$ $mN \cdot m^{-1}$ a similar interpretation is impossible at high amphotericin B mole fractions, because of desorption in the aqueous subphase. Nevertheless, up to 0.6 mole fraction the slope of the curve increases and, assuming the incompressibility of the sterol, the calculation shows that amphotericin B areas are respectively 0.81 nm^2 and0.92 nm^2 for the

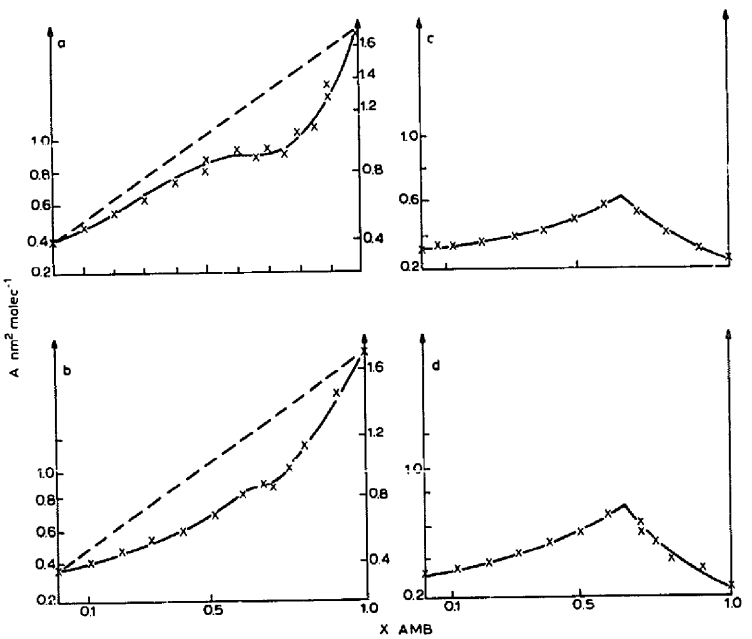


Fig. 6. Mean area per molecule versus mole fraction of amphotericin B (AMB). (a) Amphotericin B/cholesterol mixed monolayer at $P = 5$ $mN \cdot m^{-1}$; (b) amphotericin B/ergosterol mixed monolayer at $P = 5$ $mN \cdot m^{-1}$; (c) amphotericin B/cholesterol mixed monolayer at $P = 20$ $mN \cdot m^{-1}$; (d) amphotericin B/ergosterol mixed monolayer at $P = 20$ $mN \cdot m^{-1}$.

amphotericin B/cholesterol (Fig. 6c) and amphotericin B/ergosterol mixtures (Fig. 6d), instead of 0.25 nm^2 as in the case of the pure amphotericin B. Thus, these features are in agreement with what was observed at low pressure: that is, that the presence of sterol prevents the desorption of amphotericin B, probably because of an interaction between amphotericin B and sterol. A maximum area per molecule is observed for the molar fraction of amphotericin B o f0.66. It is concluded that an amphotericin B–sterol complex with a 2 : 1 stoichiometry is formed. Because the desorption of amphotericin B is smaller with ergosterol than with cholesterol, we can conclude that the amphotericin B–ergosterol interaction is larger than the amphotericin B–cholesterol interaction.

In order to ascertain the location and nature of the interaction, amphotericin B was replaced by amphotericin B methyl ester. It has been hypothesized that the interaction with sterol is different for amphotericin B and amphotericin B methyl ester [2]. The amphotericin B methyl ester/cholesterol mixture isotherms (*P-A*) are plotted in Fig. 7. The amphotericin B methyl ester isotherm (*P-A*) resembles that of amphotericin B but the pressure of the plateau is higher (13 $mN \cdot m^{-1}$) and it is impossible to compress as far as 20 $mN \cdot m^{-1}$ because the molecules become desorbed due to the charged polar group of the amphotericin B methyl ester. This strong desorption makes the interpretation of amphotericin B methyl

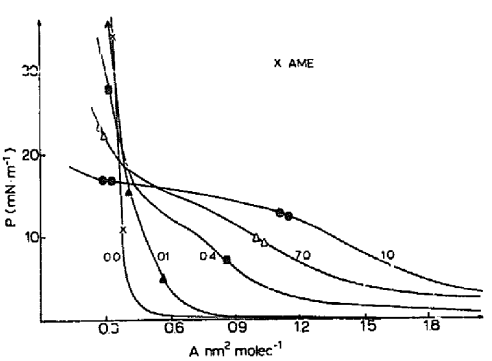


Fig. 7. Surface pressure versus mean area per molecule isotherms of amphotericin B methyl ester (AME)/cholesterol mixed monolayer spread on 10^{-1} $mol \cdot l^{-1}$ NaCl at pH 5.6 and $t = 22°C$. X = mole fraction.

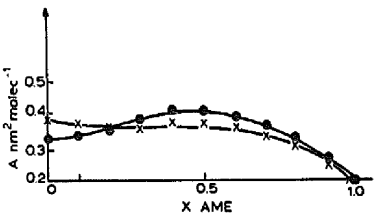


Fig. 8. Mean area per molecule versus mole fraction of amphotericin B methyl ester (AME) at $P = 20$ $mN \cdot m^{-1}$ of mixed monolayer: (a) amphotericin B-methyl ester/cholesterol (*); (b) amphotericin B methyl ester/ergosterol (●).

ester/sterol mixture isotherms difficult. Nevertheless the curve area versus the mole fraction of the amphotericin B methyl ester/cholesterol mixtures (Fig. 8a) showed that at $P = 20$ $mN \cdot m^{-1}$ the mean molecular area decreases while the amphotericin B methyl ester mole fraction decreases, when it should increase if there was no desorption. The same effect, but of smaller magnitude, was observed with ergosterol.

We can conclude that if there is an interaction, this interaction is weaker with amphotericin B methyl ester than with amphotericin B, but stronger with ergosterol than with cholesterol.

'Pseudo-binary' mixtures. It is interesting to examine the behaviour of the amphotericin B–sterol complex in the presence of egg phosphatidylcholine, under conditions close to the composition of biological membrane. 'Pseudo-binary' mixtures were studied where the composition of the complex was kept constant (0.66) and the mole fraction of egg phosphatidylcholine was variable.

The isotherms of these mixtures (amphotericin B–cholesterol)/egg-phosphatidylcholine are represented in Fig. 9). The curves with ergosterol are very similar. At pressures below 15 $mN \cdot m^{-1}$ the complexity of the isotherms was such that interpretation was very difficult. At higher pressures we could expect that the isotherms of the different mixtures would be recorded between the pure complex and pure egg-phosphatidylcholine isotherms. However, the addition of a very small amount of egg phosphatidylcholine to the complex (mole fraction of complex 0.95) drastically modified the trend of the curves. A very small mean area per molecule was obtained simultaneously with a long plateau, probably due to desorption of

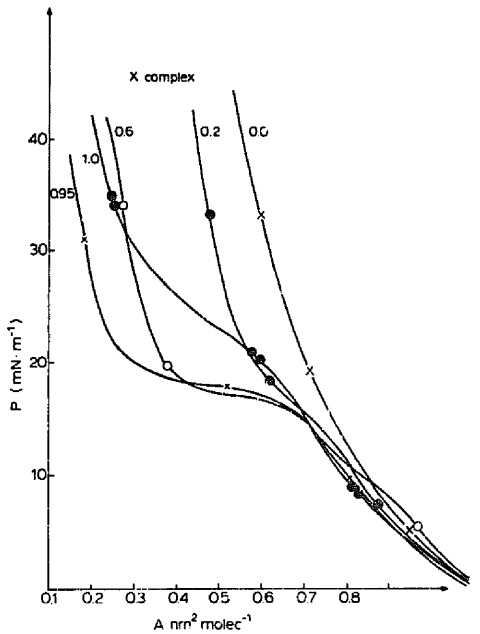


Fig. 9. Surface pressure versus mean area per molecule isotherms of 'pseudo-binary' mixture of amphotericin B–cholesterol (2:1)/egg phosphatidylcholine. X = mole fraction.

the complex or of its constituents. This desorption is clearly seen in Fig. 10 where the mean area per molecule versus the mole fraction of complex at

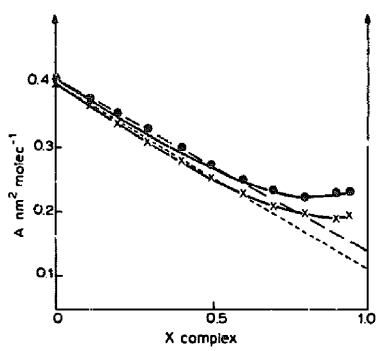


Fig. 10. Mean are per molecule versus mole fraction of the complexes at $P = 20$ mN·m^{-1} of mixed monolayer: (a) amphotericin B–cholesterol (2:1)/egg phosphatidylcholine (×); (b) amphotericin B–ergosterol (2:1)/egg phosphatidylcholine (●).

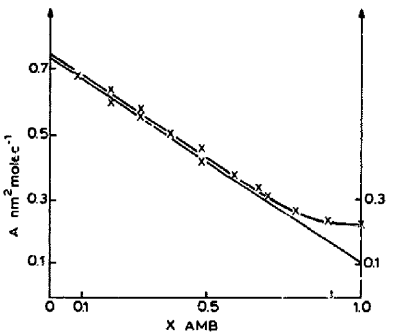


Fig. 11. Mean area per molecule versus mole fraction of amphotericin B (AMB) at $P = 20$ mN·m^{-1} of amphotericin B/egg phosphatidylcholine mixed monolayer.

$P = 20$ mN·m^{-1} is reported. Indeed, for small mole fractions all the experimental points are located on a straight line. The area per molecule extrapolated from this line to mole fraction 1 is precisely one-third of the area per molecule of the sterol, suggesting a 2:1 stoichiometry in the complex. We conclude that almost all the molecules of amphotericin B are desorbed except at high mole fraction of the complex. Thus with egg phosphatidylcholine the complex became dissociated. To determine which interaction (egg phosphatidylcholine–amphotericin B or egg phosphatidylcholine–sterol) competes with the formation of the amphotericin B–sterol complex, a study of the two binary mixtures egg phosphatidylcholine/amphotericin B and egg phosphatidylcholine/sterol was also performed.

Egg phosphatidylcholine/amphotericin B mixtures and sterol/egg phosphatidylcholine mixtures. The isotherms of the first set of mixtures were recorded and the curve mean area versus amphotericin B mole fraction at $P = 20$ mN·m^{-1} is plotted in Fig. 11. All the experimental points are located on a straight line which can be extrapolated to 0.1 nm^2 for pure amphotericin B, an area per molecule that is much too small for the antibiotic molecule. It is interpreted that, except for high mole fractions, amphotericin B is practically completely desorbed from the monolayer. Therefore the interaction between amphotericin B and egg phosphatidylcholine is very weak, in agreement with the results of Demel et al. [11].

Analogous investigations using egg phosphatidylcholine/sterol mixtures have been performed by many authors, who described a condensation effect, leading to a complex with a stoichiometry of 1:1 [17]. We observed that the same kind of results is obtained with ergosterol, but the interaction is weaker [18].

Discussion

The simultaneous radiolabelling and surface pressure experiments show clearly a penetration of amphotericin B in phospholipid/sterol monolayers. This penetration is limited and is probably followed by a simple adsorption of the antibiotic as oligomers on the polar surface of the monolayer, which was confirmed by the compression isotherms of the amphotericin B–sterol/egg phosphatidylcholine binary mixtures which showed an expulsion of the amphotericin B beyond a surface pressure close to 20 $mN \cdot m^{-1}$ which is precisely the maximum pressure reached in the penetration experiments.

To check whether or not an amphotericin B/sterol monolayer spread on the water surface is identical to a pure sterol monolayer in which amphotericin B has penetrated from the subphase, a monolayer obtained by penetration of amphotericin B such those used in the first part of this work was rapidly decompressed and then compressed again. We observed that the curve exhibited the same trend as the isotherm corresponding to a 2:1 amphotericin B/sterol mixture spread on water. This fact is consonant with the identity of spread and adsorbed monolayers as shown previously by Ter Minassian Saraga [19].

From these data, it is possible to make a hypothesis concerning the mechanism of interaction of polyene with monolayers. In the concentration range studied in penetration experiments ($5 \cdot 10^{-7}$–$5 \cdot 10^{-6}$ $mol \cdot l^{-1}$), polyenes are not in monomeric solution in the subphase, but mainly in oligomeric form [20], the hydrophobic heptaene backbones of the macrolide ring being in close contact. In order to be spread at the air/water interface the oligomers dissociate. Polyene molecules then adopt a 'lying' position, exposing their hydrophobic heptaene backbones to air. The assumption that, in order to penetrate, polyene in solution must first adopt a 'lying' position accounts for the great sensitivity of penetration to the initial pressure of the monolayer. With increasing surface concentration and pressure, polyene molecules tend to adopt an 'erect' position, again forming oligomers in the monolayers, but of reverse structure, that is, with the hydrophilic moiety of the macrolide ring facing each other. The analysis of polarized absorption data published by Ockman [13] shows that this 'erect' position is favored by the presence of sterol. Moreover, from the continuous accumulation of molecules at the interface in spite of the stabilization of the surface pressure, it can be assumed that amphotericin B molecules can not only penetrate into the membrane but also be adsorbed on it.

Our experimental results suggest furthermore the formation of an amphotericin B–sterol complex with a 2:1 stoichiometry, consonant with the formation of a dimer with amphotericin B molecules in 'erect' form whose association is energetically favored. It would be interesting to examine whether this can be confirmed from the penetration experiments. Indeed the corresponding pressure-concentration curves (Fig. 2) resemble Langmuir adsorption isotherms, at variance with the adsorbed amount versus concentration curves. It could be assumed that the former type would take into account only the penetration phenomenon while the latter would describe the global situation resulting from penetration and adsorption. Thus, the pressure-concentration curves, dealing with the penetration only, should allow a choice between a 1:1 or 2:1 binding model and a calculation of the affinity, but the Langmuir adsorption isotherms concern the penetrated amount and not the pressure, which is an intensive parameter, the variation of which is generally not proportional to penetrated amounts. Therefore calculations are physically significant only in the concentration range in which pressure is proportional to the amount of amphotericin B present in the surface, assuming that the penetration is prominent in this case.

We checked that this proportionality is observed for the lowest concentration only up to about $1.5 \cdot 10^{-6}$ $mol \cdot l^{-1}$. Now, it is observed that in this range, within the accuracy in the measure-

ments. The $1/P$ versus $1/C$ or $1/C^2$ plot is not a straight line whatever the binding stoichiometry, maybe because the hypothesis according to which the adsorption is negligible at low concentration is wrong. Thus it is not possible to deduce any binding stoichiometry or affinity constant from the penetration experiments.

These monolayer studies throw some light on the data obtained concerning the binding of polyene to biological membranes, which otherwise would be difficult to interpret. Beezer and Sharma [21], measuring the binding of nystatin to yeast cells, found that this binding was not saturable up to 10^{-5} mol $\cdot l^{-1}$ and that the calculated area of the yeast plasma membrane was not large enough to accommodate the calculated surface occupied by the molecules bound at this concentration. More recently the binding of various amphotericin B derivatives to red blood cells [20] was not found to be saturable either in the range 10^{-8} to 10^{-5} mol $\cdot l^{-1}$, which can be explained if an adsorption on the membrane is combined with a penetration of antibiotic. This adsorption depends upon the amount of polyene already penetrated in the monolayers. Indeed there is very little adsorption in pure sterol/phospholipid layers as shown by the very small radioactivity found above 20 mN $\cdot$ m^{-1}. Therefore, it must be assumed that the polyene molecules are anchoring mainly on molecules already penetrated in the monolayer, in agreement with their strong tendency to self-associate. This accounts for the lack of correlation between the measured binding and the ionophoric activity in the membrane, and may explain why saturable binding sites cannot be found.

Our experimental results provide evidence, for the first time, that egg phosphatidylcholine competes for sterol with amphotericin B. Thus the large discrepancy observed among the amphotericin B/sterol stoichiometries proposed by various authors can be explained by such a competition mechanism which depends strongly on experimental conditions. However, the fact that an amphotericin B–sterol complex seems to exist only in amphotericin B/sterol monolayers and is not detectable in the presence of phospholipids tends to indicate that, if it exists, this complex is very loose, in contrast to filipin–sterol complexes, and its formation is very much dependent on the other membrane constituents.

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